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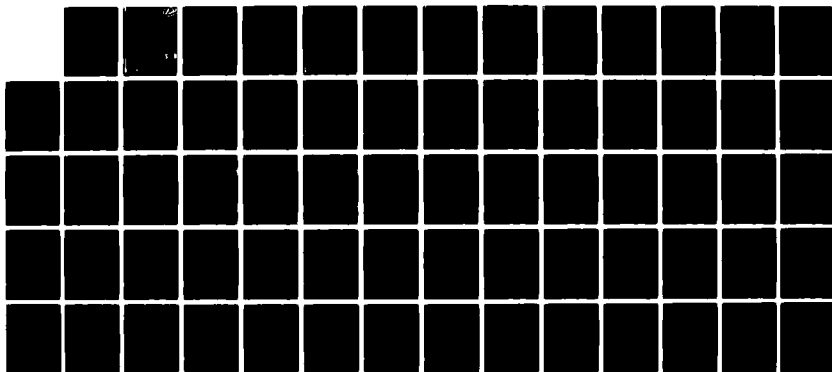
EKTACYTOMETRY: INSTRUMENTATION AND APPLICATIONS IN RED 1/1
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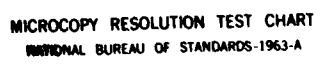
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EKTACYTOMETRY:
INSTRUMENTATION AND APPLICATIONS IN RED BLOOD CELL
PRESERVATION STUDIES

by

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10 May 1982

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freezing in 40% W/V glycerol had any effect on the ektacytometry measurements. We determined that it was necessary to maintain an isotonic environment, since the red blood cells respond differently to shear stress under hypertonic or hypotonic conditions. The most unexpected observation was an absence of correlation could be established between deformability as determined by ektacytometry and 24-hour posttransfusion survival of ^{51}Cr -labeled red blood cells previously frozen and washed. This can be partly attributed to the large variations in data among individual red blood cell samples and to the day-to-day imprecision of the method itself.

Ektacytometric analysis showed a higher shear-induced elongation of young red cells (neocyte-enriched populations) in comparison to old cells. The two populations were fractionated by density centrifugation.

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A. ABSTRACT

↓
An ektacytometric instrumentation system was set-up in the laboratory and its usefulness in red blood cell (RBC) preservation studies was evaluated. Ektacytometry provides a measure of cell deformability by determining the elongation of the cells under fluid shear stress using laser diffractometry and image analysis.

In order to objectively assess the data obtained with red blood cell samples, it was necessary to establish that neither white blood cell concentration nor freezing in 40% W/V glycerol had any effect on the ektacytometry measurements. We determined that it was necessary to maintain an isotonic environment, since the red blood cells respond differently to shear stress under hypertonic or hypotonic conditions. The most unexpected observation was an absence of correlation could be established between deformability as determined by ektacytometry and 24-hour posttransfusion survival of ^{51}Cr -labeled red blood cells previously frozen and washed. This can be partly attributed to the large variations in data among individual red blood cell samples and to the day-to-day imprecision of the method itself.

Ektacytometric analysis showed a higher shear-induced elongation of young red cells (neocyte-enriched populations) in comparison to old cells. The two populations were fractionated by density centrifugation. ↗

B. EKTACYTOMETRY

1. Principle.

Ektacytometry as developed by Bessis and Mohandas¹ involves the application of fluid shear stress on cells placed in a concentric cylinder viscometer and the resulting cellular deformation is measured using laser diffractometry. The diffraction pattern from a dilute suspension of cells is obtained by passing a beam of laser light through the suspension. Under the action of various shear stress conditions, the cells are elongated and, therefore, their diffraction image changes from circular to elliptical shape. By monitoring the diffraction shape changes, one can measure the elongation of the cells, and from this deduce their deformability.

2. The Ektacytometer.

A Bessis-Mohandas ektacytometer was constructed basically as described by these authors,¹ with some modifications of our own. Figures 1 and 2 show the general instrumentation assembly. The light beam is generated by a Helium-Neon laser (Spectra Physics, Model 124B), and by means of two glass prisms traverses radially the narrow chamber gap (0.24 mm) which is formed between the inner and outer concentric cylinders of a rotational type viscometer. The inner cylinder (82 mm in diameter) remains stationary whereas the outer cylinder rotates under the control of the Haake Rotovisco RV3 motor controller. The revolution of the outer cylinder increases gradually from zero to 100 rpm corresponding to values of shear stress which range from zero to 45 Pascal units ($\text{dyne/cm}^2/10$)

using 25% dextran solution in phosphate-buffered saline (PBS). The diffraction pattern generated by the cell suspension in the cylindrical annulus (gap) can be visualized on a vertical dark panel perpendicular to the laser beam. The dimensions of the diffraction ring are approximated by two silicon photodiodes (United Detector Technology, PIN 3DP) mounted in the vertical and horizontal slots of the detection screen at distances corresponding to 30° angle (Figure 2). The output current of each photodiode, which exhibits a linear relationship to light intensity, is converted into a voltage signal by an amplifier (United Detector Technology, Model 101A) with a gain of 10^7 V/A. The voltage is recorded manually.

From the recorded voltages A (vertical photodiode) and B (horizontal photodiode), the light intensity ratio (LIR) is estimated by²:

$$(LIR) = A-B/A+B$$

At zero shear stress, the circular diffraction pattern produces equal light intensities at the two photodiodes and, therefore, LIR is equal to zero.

An alternate method for estimation of the dimensions of the ellipse produced by the diffraction pattern involves digitization of the image by a Reticon camera, storage of the data on a floppy disc, and subsequent estimation of the dimensions of the two ellipse axes by computer (Hewlett-Packard).

3. General Procedure.

To measure red blood cell deformability, 100 μ l of packed red cells with a total count of 4.5×10^8 are suspended in 15 ml of an isotonic PBS solution containing 9.0 g NaCl, 0.2 g KCl, 1.15 g Na_2PO_4 , 0.2 g

KH_2PO_4 , 0.1 g MgCl_2 , 6 H_2O , 0.1 g CaCl_2 , 250 g dextran (69-90) and 1 liter water. Dextran is used to increase the viscosity of the suspension medium. Ten ml of the red blood cell suspension is added to the ektacytometer chamber and the cells are subjected sequentially to revolutions of 9, 18, 35, and 72 rpm corresponding to shear stress values of 5.2, 9.8, 7.5, and 31.0 Pascal units, respectively. The A and B voltages are recorded, and the LIR values are estimated for each revolution setting. A plot of $(1/\text{LIR})$ vs $(1/\text{Shear Stress})$ produces a straight line, the slope of which is estimated by linear regression. The slope reflects the magnitude of elongation of the red blood cell as a function of shear stress and, therefore, provides a measure of deformability.

C. EXPERIMENTAL

1. Reproducibility of Measurements.

A fresh frozen and a two-day-old frozen human red blood cell sample were used as "controls" to test the reproducibility of the ektacytometer measurements over a 105-day period. Figure 3 shows the LIR values plotted vs. shear stress. It can be seen that for each shear stress setting, the standard deviation of the measurements increases with the applied shear stress. Thus, the errors are larger at the LIR maximum. The day-to-day variation of the LIR maximum for the two control samples is approximately $\pm 8\%$ as illustrated in Figures 4 and 5. Since, for a complete discrimination between two unknown samples four standard deviations are needed, it appears that differences in LIR max values of more than 32% are necessary to call two samples different with confidence.

The standard deviation of the same-day variation in the measurement of the same sample is of the order of 3% (Figure 6) and, therefore, differences above 12% are required to compare samples analyzed the same day.

2. Treatment of Human Red Blood Cells With Hypotonic and Hypertonic Solutions and Glutaraldehyde Fixation

In order to study the effect of shape and volume change on shear stress elongation, human red blood cells were incubated in a hypotonic phosphate-buffered solution containing 0.6% NaCl (pH 7.4), in an isotonic solution containing 0.9% NaCl (pH 7.4), and in a hypertonic

solution containing 1.5% NaCl (pH 7.4). The morphologic changes produced by these solutions are reported in Table 1.

Figure 7 shows that a greater increase in LIR values was observed with the hypotonically swollen cells than in the control (isotonic). Crenated cells exhibited negative LIR values. The ektacytometry data were obtained while the cells were in these solutions. Negative LIR values were also observed for glutaraldehyde-fixed cells.

In another series of experiments, fresh and five-day-old blood samples collected in EDTA were placed on a Sigma cell cellulose column (equilibrated with PBS) to remove the white blood cells. The erythrocytes were obtained by centrifugation and resuspended in various concentrations (i.e., 0.7, 0.8, 0.9, 1.0 and 1.6%) of NaCl to approximately 30% concentration by volume. The cells were left to stand for at least 1 hour at room temperature in these solutions. After this period small volumes of the suspension (40-80 μ l) were mixed with 10 ml of isotonic PBS-dextran solution for the ektacytometry studies.

It may be seen from Figures 8, 9 and 10 that the erythrocytes behave similarly in the ektacytometry test after resuspension in the isotonic solution. Thus, it can be concluded that any volume changes imparted by non-isotonic solutions on the cells prior to the test are reversible within the limits mentioned above.

3. Effect of White Blood Cell Concentration on Ektacytometric Red Blood Cell Measurements.

Twenty-two human red blood cell samples were prepared where the concentration of white blood cells varied, and ektacytometric analysis

was performed to examine their effect on the diffraction data. Figure 11 demonstrates that there is no correlation between white blood cell counts in the sample and red blood cell ektacytometric data as expressed by the slope of the plot of $1/LIR$ vs. $1/Shear\ Stress$. Thus, white blood cell contamination would not be expected to interfere with the ektacytometry of the red blood cells.

4. Comparison of Fresh and Frozen Red Blood Cells.

In this experiment fresh human red blood cells were compared to cells frozen in the presence of 40% W/V glycerol and thawed and washed. As Figure 12 illustrates, no differences in the ektacytometric data were observed in these two samples.

5. Ektacytometry of Reticulocyte-Enriched Fractions.

Blood samples were obtained from healthy volunteers in acid-citrate-dextrose (ACD, NIH, Formula A). These samples were subjected to phthalate ester fractionation in 50 ml plastic test tubes to separate the young red blood cells (neocytes) and old red blood cells (gerocytes).³ The young red blood cells were obtained using low density phthalate solution ($e = 1.0808$ and 1.0872) fractionation, and the old using high density phthalate ($e = 1.1120$).

Figure 13 shows that the cells in the low density phthalate fraction (enriched with 28.7% reticulocytes) are more deformable than those in the high density one. The effect of phthalate itself on red blood cell deformability is unknown.

Similar results were obtained when neocytes collected on the Fenwal

CS-3000 granulocyte chamber were compared to the red blood cells in the predonation blood sample (Figure 14). However, when the platelet chamber was used with the same separator, the neocyte-enriched fraction behaved similarly to the predonation sample (Figure 15).

6. Ektacytometric Analysis of Autotransfused ^{51}Cr -labeled Red blood Cells and Correlation with 24-Hour Posttransfusion Survival

The objective of this study was to examine the possible correlation between 24-hour posttransfusion survival of autotransfused ^{51}Cr -labeled red blood cells and ektacytometry data. The red blood cell samples were analyzed as fresh and after frozen storage and chromium labeling. Two types of plots were obtained, one of % survival vs. LIR max and the other % survival vs. log of the slope of the plot of $1/\text{LIR}$ vs. $1/\text{Shear Stress}$.

Figures 16 and 17 exhibit the scattergram of the individual fresh samples. The standard deviations of the ektacytometry data were much higher than would be expected from the day-to-day instrumental error, and were probably due to individual sample variation in deformability. The ektacytometry data showed even higher variability in chromium-labeled preserved red cells (Figures 18 and 19) than in the fresh red blood cells, but no correlations with 24-hour posttransfusion survivals could be demonstrated.

The above results illustrate that ektacytometry in its present form cannot be used as a predictor of 24-hour posttransfusion survival value of preserved red blood cells.

7. Effect of Red Blood Cell Concentration on Ektacytometry Data.

In a series of three experiments the concentration of human red blood cells was varied in the ektacytometry chamber in order to assess the possible effects of cell interactions on their deformability. Figures 20, 21 and 22 show that the red blood cell concentrations had a pronounced effect on the LIR under increasing shear stress. A linear relationship was obtained between LIR max and cell concentration (Figure 23) with the deformability decreasing as the red blood cell concentration increases. For this reason, all the ektacytometry data which were used for comparative reasons in this report were obtained at a fixed cell concentration.

8. Effect of Shear Stress on Red Blood Cell Hemolysis.

The objective of the RBC hemolysis experiments was to examine if the ektacytometry rotor exhibits sufficient shear stress to induce lysis in erythrocytes which may result in a decrease of their concentration. To this end, two duplicate samples were prepared from each blood sample, each containing 2.0×10^8 cells in 10 ml of Dextran-PBS suspension. One of the samples was placed in the ektacytometer chamber for measurements and the duplicate sample was not subjected to any shear stress at all. After the test, both samples were centrifuged at 2000 rpm for 15 minutes. The supernatant was then removed and stored at 4 C until hemoglobin analysis could be made. The hemoglobin content was determined by using the tetramethylbenzidine (TMB) assay.

The results of these experiments are shown in Table II. The mean hemoglobin concentration in the supernatants of the control RBC (i.e. in

the absence of shear stress) was 7.44 ± 2.56 mg% and that of the post stress supernatants 8.80 ± 2.93 mg%. These differences are too small to have an effect on RBC concentration of such magnitude as to effect the ektacytometry data.

D. CONCLUSIONS

The most significant conclusion from this work is that ektacytometry cannot be used to predict the 24-hour posttransfusion survival of preserved human red blood cells, because of the wide variations in ektacytometric behavior of various red blood cell samples as well as instrumental errors.

Neither the cryopreservation process (40% W/V glycerol) nor the white blood cell concentration affected the ektacytometric measurement of red blood cell samples. Hypotonic and hypertonic effects were observed, but these were reversible when the cells were resuspended in isotonic solutions.

So far, experiments performed with density fractionated red blood cell samples show some correlation between shear-induced cell elongation and red blood cell age.

E. REFERENCES

1. Bessis, M., and Mohandas, N. (1975) Blood Cells 1:307-313.
2. Bessis, M., Mohandas, N., and Feo, C. (1980) Blood Cells 6:315-327.
3. Danon, D. and Marikovsky, Y. (1964) J Lab Clin Med 64:668-674.

F. APPENDIX: OPERATIONAL INSTRUCTIONS

EKTACYTOMETRY

1. Sample Preparation:

All samples are stored in the 4 C refrigerator. A "standard" sample is measured each day for quality control. This sample, the P₅₀ "fresh" control, is kept in a 4 C refrigerator. Record the batch number of this sample each day.

a. Cell Counts (4th Floor).

A Coulter count on each sample is necessary to normalize the RBC concentration. The cells are diluted twice, 1/500 and then 1/100, for a total dilution of 1/50,000. The dilution is measured three times to get the mean RBC count and mean cell volume (MCV).

b. Phosphate-Buffered Saline and Dextran.

To a 400 ml beaker, add 25 g dextran and 100 ml PBS. Stir until all dextran has dissolved. (Dextran, a polysaccharide, is used to increase the viscosity of the suspension medium). This solution is poured into a 100 ml flask.

c. The blood is diluted in dextran solution to obtain a concentration of 20,000 RBC/ μ l

Example: Coulter count is 4.0×10^6 cells/ λ (20,000 cells/ λ)(10,000 λ)
 $= 2.0 \times 10^8$ cells

$$\frac{2.0 \times 10^8 \text{ cells}}{4.0 \times 10^6 \text{ cells}} = 50\lambda$$

Then 50 μ l of whole blood (2×10^8 cells) is suspended in 10 ml of the medium in a 20 ml Coulter cuvette.

d. Injection into Cylinder.

Sample preparation (Coulter count, dilution) should take about 10 minutes. The 10 ml RBC suspension is injected into viscometer using a 10 cc syringe with an 18 g needle. Measurements can now be recorded.

2. Beam Alignment:

The Helium-Neon laser light beam is deflected by two mirrors outside the chamber and two glass prisms inside the chamber to pass radially through the chamber gap. The beam then hits the detection screen at a specific point. The beam was previously centered on the screen but due to scattered light (from scratches on the chamber) it was moved one inch off center. The beam must be lined up in this position before running each sample.

To check beam alignment: A green test tube stopper has been placed in the screen to mark the correct position. The laser beam must hit the center of this point. The beam can be aligned horizontally by moving the ektacytometer right or left. This is sufficient for minor adjustments. Additional adjustments can be made by moving the mirrors and/or the laser itself. Vertical beam alignment can be accomplished by moving the mirrors up or down. The laser can also be raised up or down if necessary. For normal minor adjustments, move the ektacytometer right or left and the mirrors up or down.

When beam is centered, check background voltage readings (voltmeter

setting: 2.5 DCV). The values should be less than 0.1 volt at the A2 and B1 photodiodes. If readings are high: examine chamber, prisms, and mirrors; look for dust, scratches, or fingerprints; clean if necessary.

3. Operation of Ektacytometer:

All power switches are turned on. Main switch (supplies all equipment), two switches are on amplifier, and "LINE" button of Haake motor controller. A small attenuator switch on top of the Spectra-Physics laser is opened to allow emission of the laser beam. Shut off overhead light in room (only use small desk lamp).

a. Motor Control.

The Haake motor control is operated in the "FIX" mode. The "STOP" button can be used at any time. Four measurements are made: 9, 18, 36, 72 rpm (buttons are labeled: 90.5, 181, 362, 724). After each measurement, simply push the next higher button.

b. Amplifier and voltmeter operation.

The amplifier and power supply are both turned on. The amplifier is set on "HI SENS". The voltmeter is turned to 10 DCV setting. At each rpm, two points are plotted on graph paper. Photodiode B1 on the horizontal axis and photodiode A2 on the vertical axis. The needle of the voltmeter will fluctuate so an average value (done mentally) is recorded. Watch the needle for 15 seconds or so, and then plot the average value. Measurements take about 5-10 minutes to complete.

c. Washing Cylinder.

HANDLE WITH CARE! (avoid unwanted scratches). Remove 4 screws on top and lift cylinder out. Wash out both cylinders in the sink and

then rinse with distilled water. Flush out the injection hole with distilled water (using the 10 cc syringe). Wipe dry with 4 X 4 gauze and reassemble. Washing cylinder takes about 5-10 minutes.

Measurements should be finished after 20 minutes. Prompt measurements will minimize the effects of dextran on the red cells. Each sample requires a total time of about 45 minutes from start to finish.

4. Data Analysis:

The calculations and graphs are made on the same sheet of graph paper that has the data points on it. For each rpm, the two points are put into the equation $A-B/A+B$ to get the light intensity ratio (LIR). A graph is now made by plotting $1/LIR$ vs. $1/\text{shear stress}$. Estimate slope by linear regression. All data are transferred to a large computer data sheet which simplifies the process of storing data in the computer. The sheet of graph paper is put into the notebook according to the type of study.

5. Comments:

The ektacytometer is a recently developed prototype. The technique of measurement is not a simple step-by-step procedure. Difficulties in the apparatus occur on a regular basis. Problems that will affect measurements are:

- a. Scratches on the plexiglass cylinders
- b. Centering the laser beam in the correct position on the photodiode screen
- c. Dust on mirrors or plexiglass cylinders
- d. Misalignment of the cylinders will cause the step motor connecting pin to shear (replacement takes about 1 hour)

TABLE 1Effect of Tonicity on Red Blood Cell Indices and Morphology

<u>% NaCl, pH 7.4</u>	<u>mOsm/kg H₂O</u>	<u>MCHC (%)</u>	<u>MCV (μ^3)</u>	<u>Morphology</u>
0.9	306	36.6	90	100% Discoidal
0.6	200	29.1	103	Swollen
1.5	496	44.5	77	Crenated

TABLE IIEffect of Shear Stress on Red Blood Cell Hemolysis

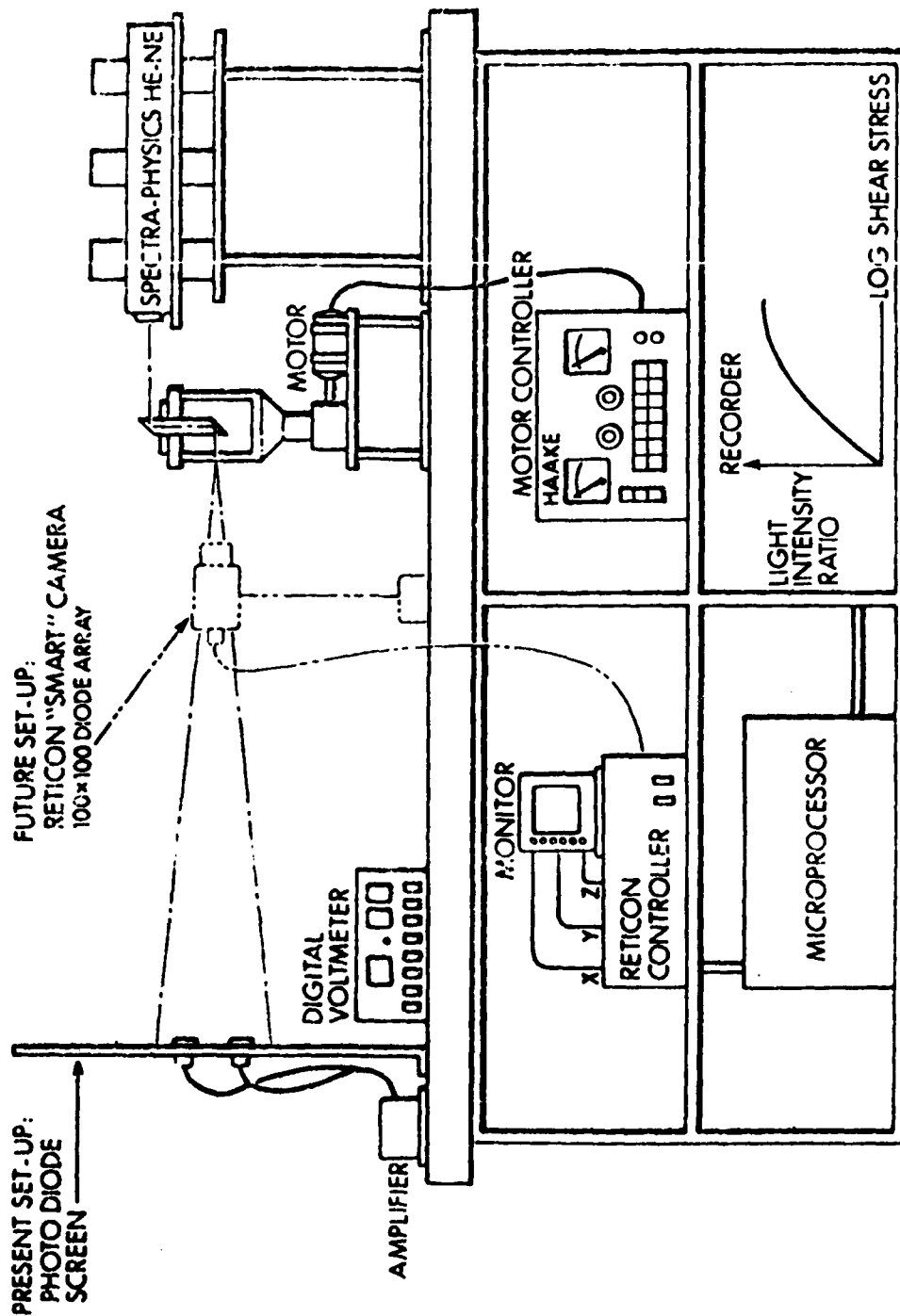
<u>Blood Sample</u>		<u>W/O Shear Stress Hemoglobin mg%</u>	<u>Post Shear Stress Hemoglobin mg%</u>
6-Day-Old Solution C Control	Post-wash	8.3	14.6
6-Day-old Solution C Control	Post-Wash	6.9	6.7
1-Day-Old NR Control	Post-wash	10.8	10.4
AT-922, 24-Hour	Post-Wash	<u>12.4</u>	<u>12.8</u>
	Mean	9.6	11.1
	SD	2.5	3.4
AT-927	Fresh	8.4	7.6
#001806	Fresh	7.8	13.8
#001807	Fresh	6.8	6.7
#001808	Fresh	5.9	6.3
#001809	Fresh	11.3	11.7
#001812	Fresh	3.1	6.4
Mostacci	Fresh	<u>8.8</u>	<u>9.1</u>
	Mean	7.4	8.8
	SD	2.6	2.9

FIGURE 1

Schematic diagram of the ektacytometer assembly at the Naval Blood Research Laboratory.

FIGURE 1

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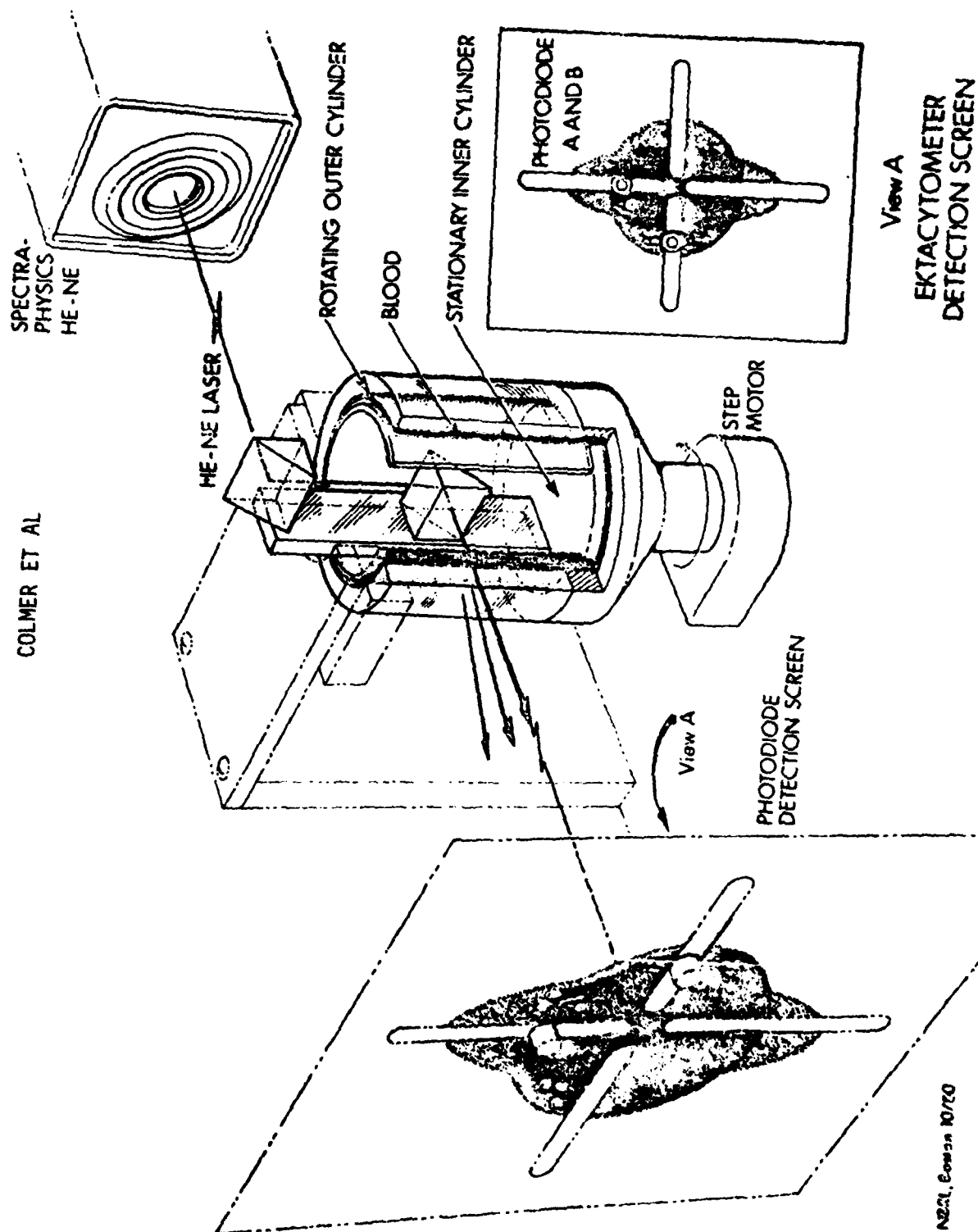


LASER EKTACYTOMETER

FIGURE 2

Schematic diagram of the ektacytometer chamber and diffraction patterns produced by red blood cells subjected to shear stress.

FIGURE 2
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FIGURE 3

Plot of LIR vs. shear stress for fresh frozen and two-day-old frozen human red blood cells analyzed over a period of 105 days.

FIGURE 3

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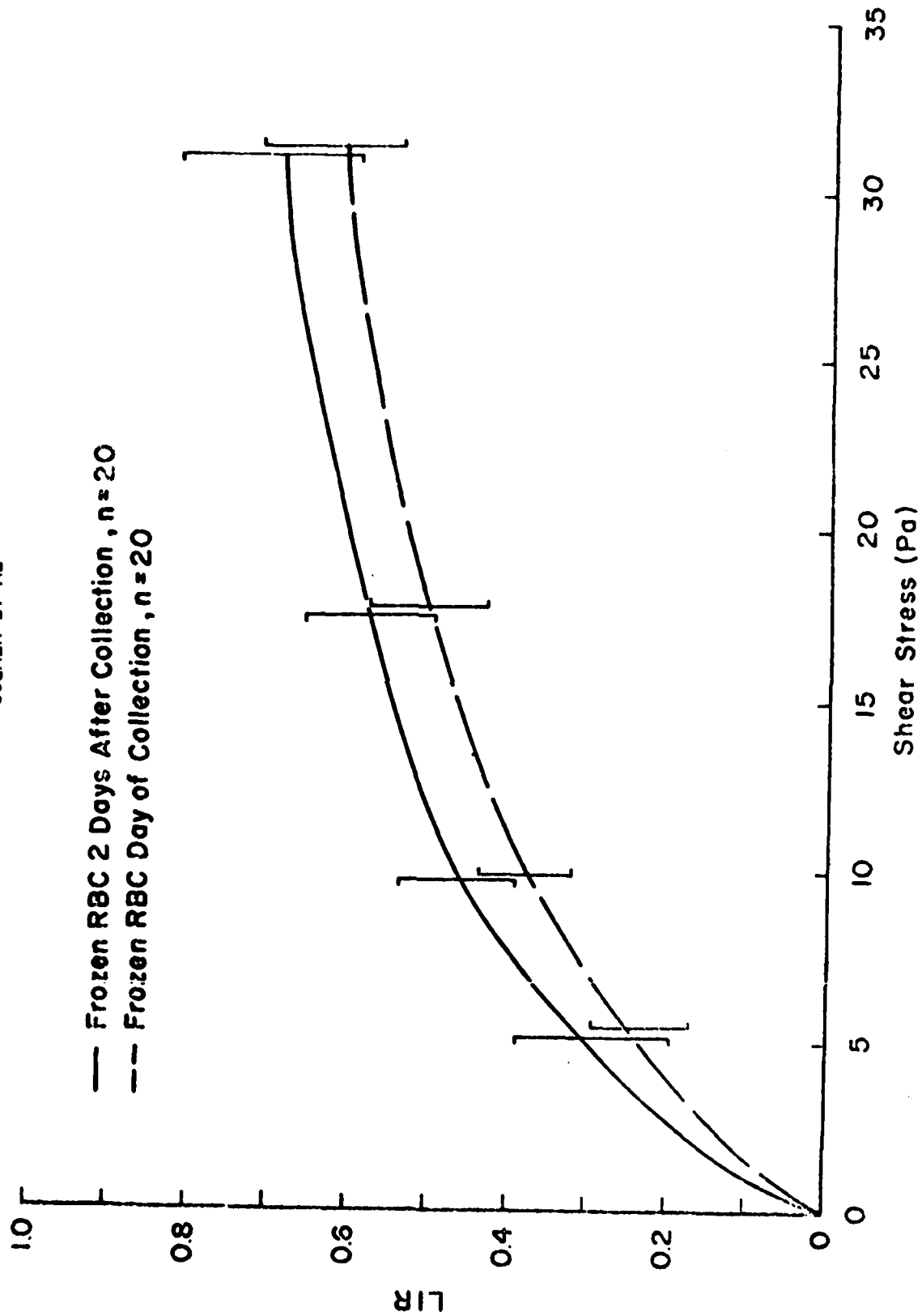


FIGURE 4

Day-to-day variation of LIR max values of fresh frozen human red blood cells.

FIGURE 4

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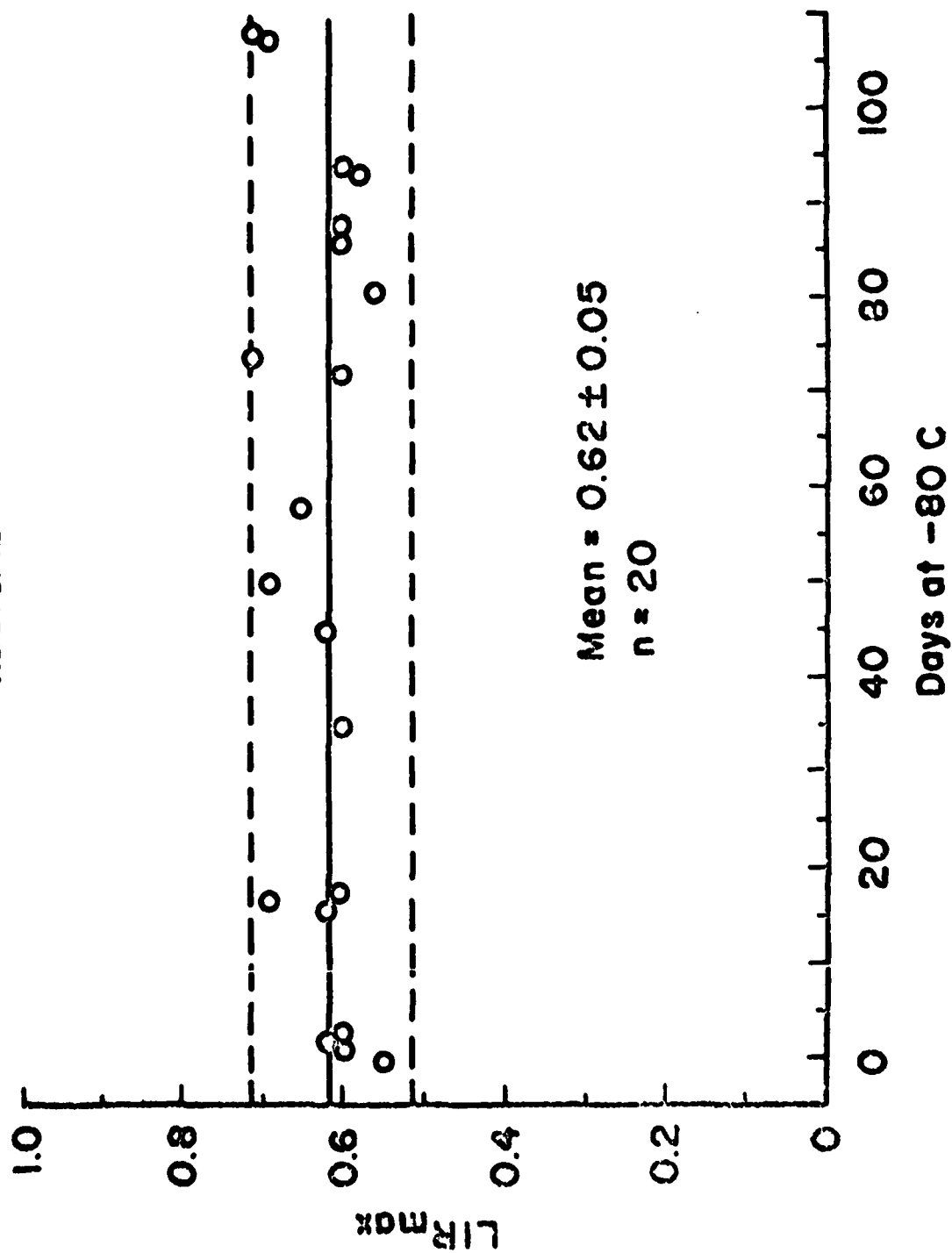


FIGURE 5

Day-to-day variation of LIR max values of two-day-old frozen human red blood cells.

FIGURE 5

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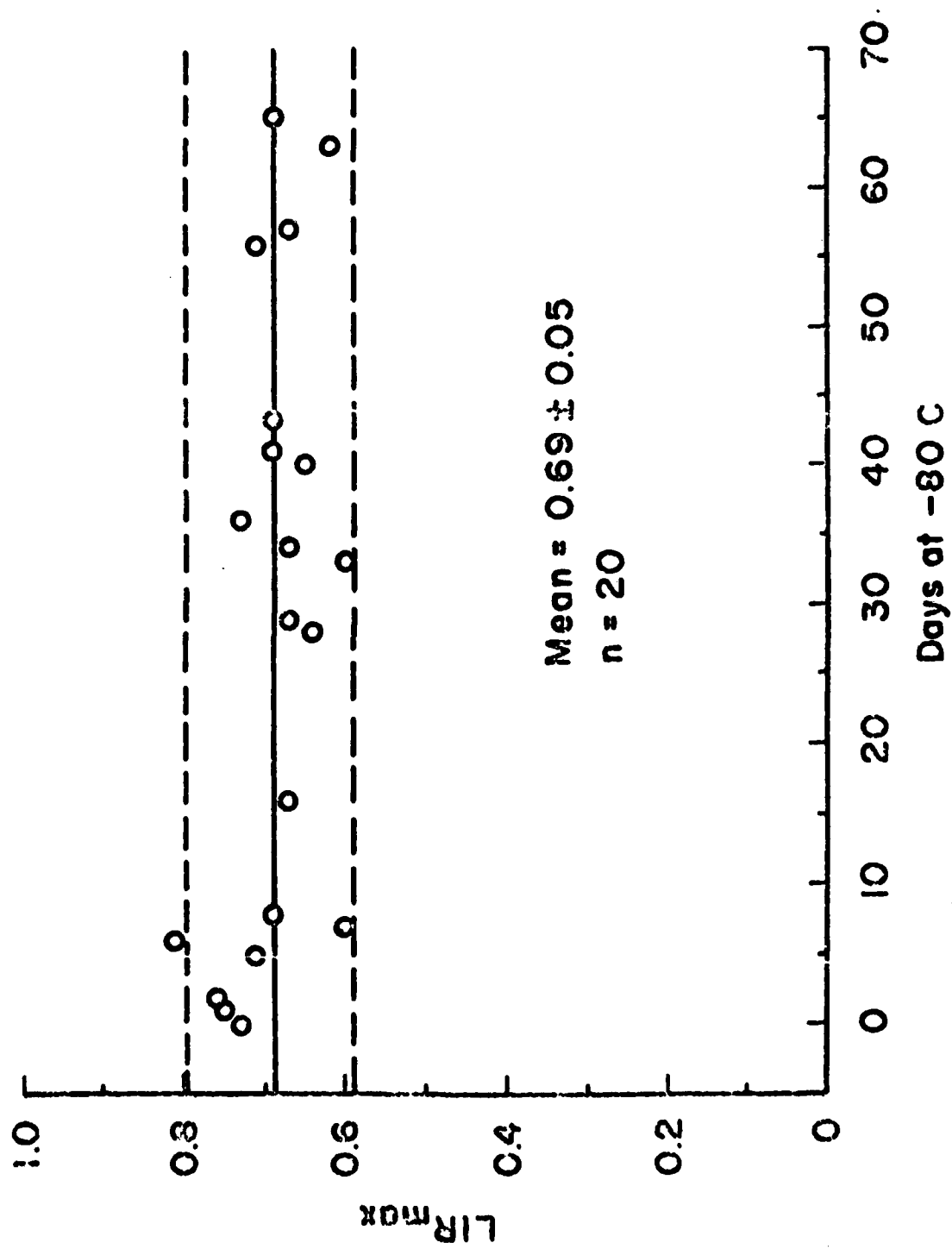


FIGURE 6

Same day reproducibility of LIR measurements vs. shear stress for the same human red blood cell sample measured three times (LIR max 0.65 \pm 0.02)

FIGURE 6

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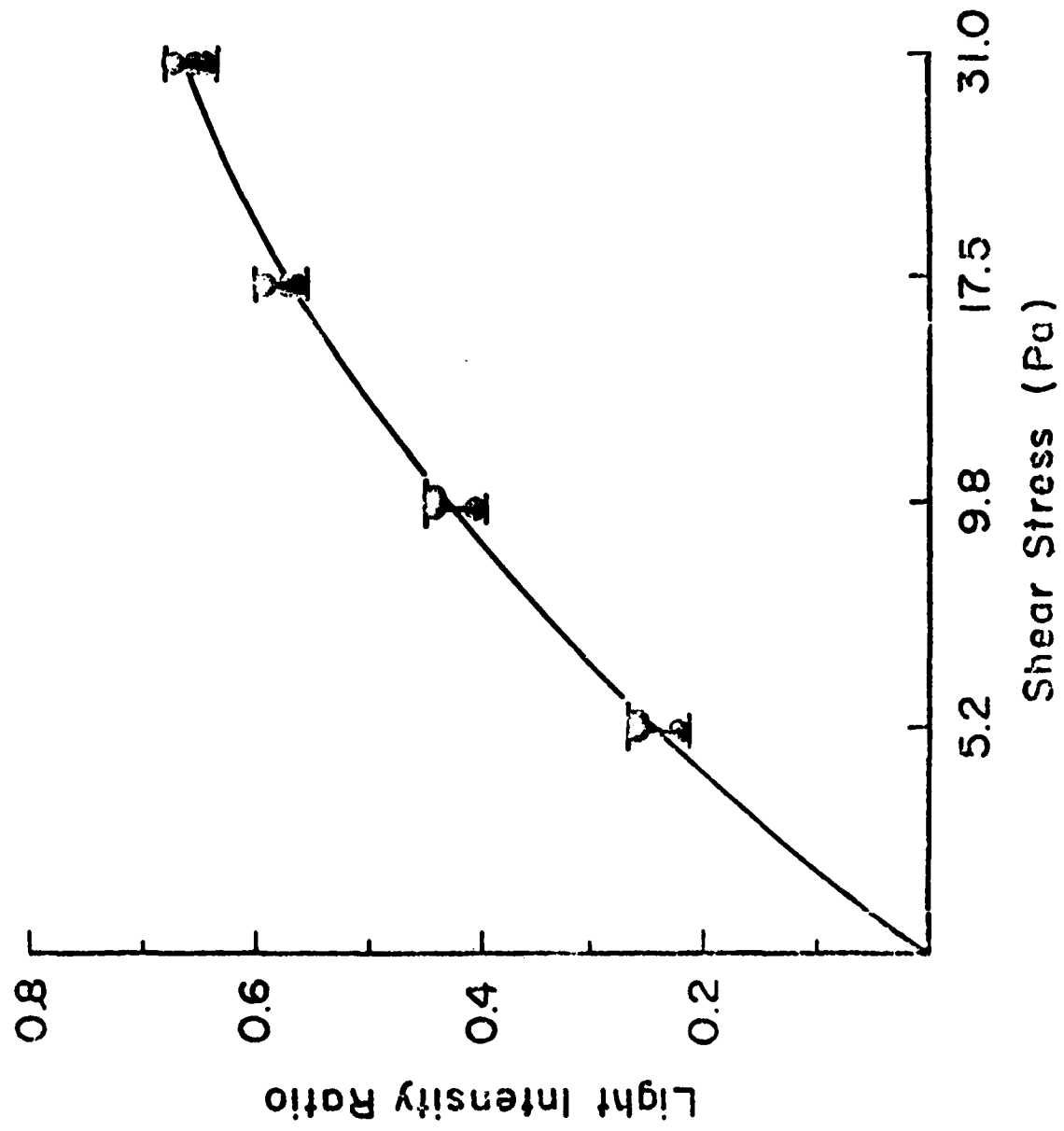


FIGURE 7

The effect of tonicity and glutaraldehyde fixation on the LIR of human red blood cells under shear stress.

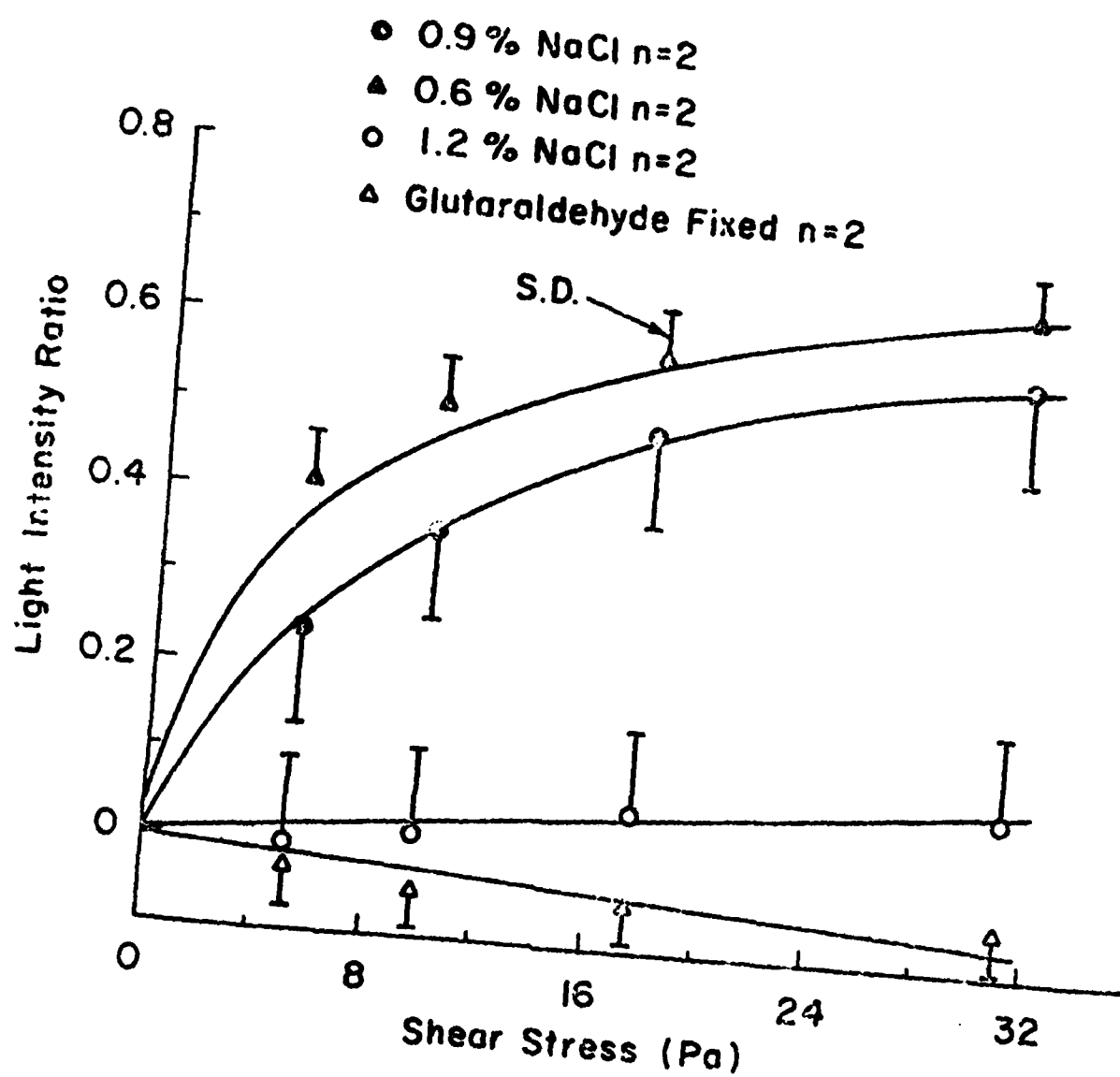


FIGURE 7

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FIGURE 8

The effect of treatment of fresh human red blood cells with various concentrations of NaCl followed by resuspension in isotonic PBS-dextran solutions on the LIR under shear stress.

FIGURE 8

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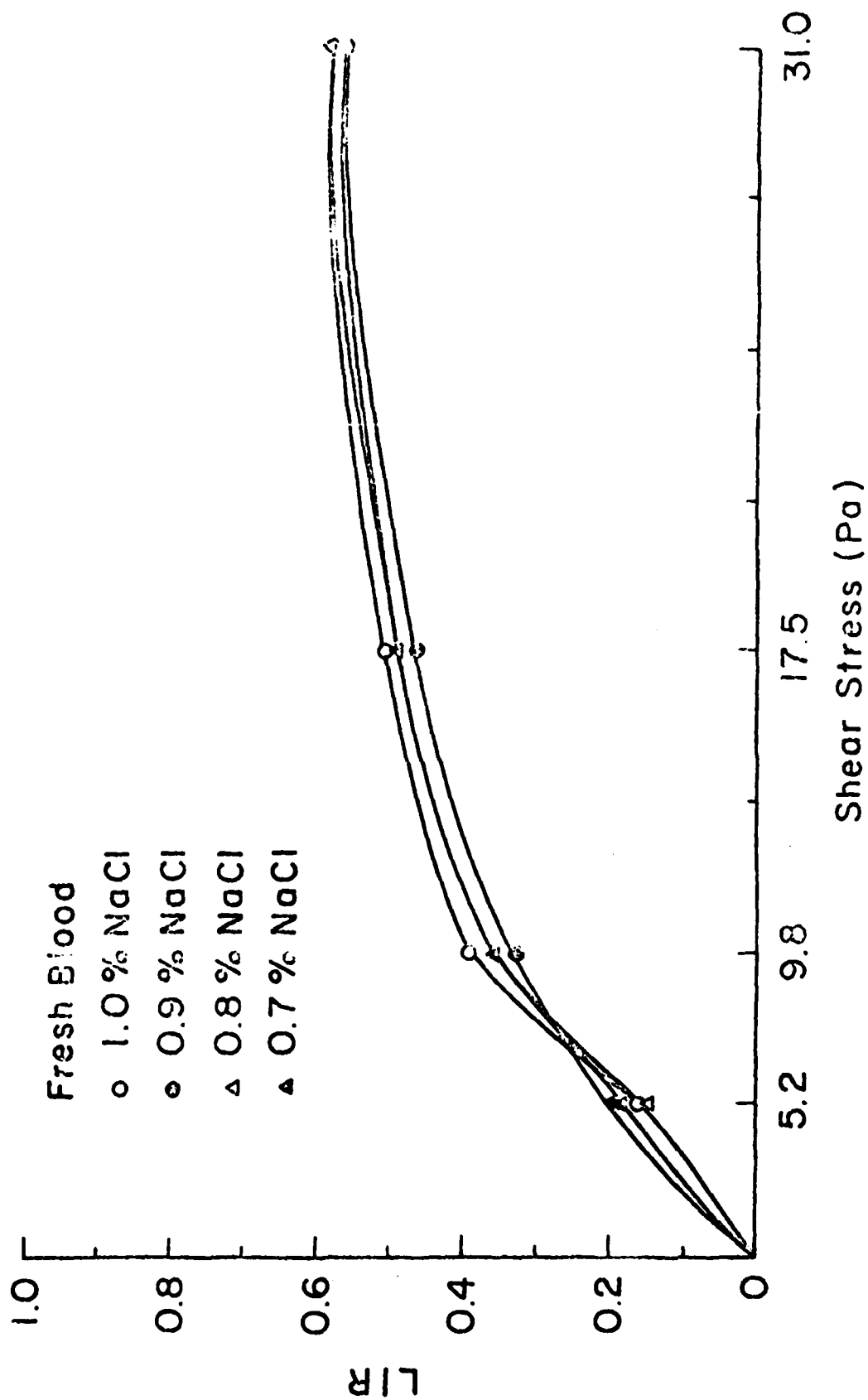


FIGURE 9

The effect of treatment of five-day-old human red blood cells with various concentrations of NaCl followed by resuspension in isotonic PBS-dextran solutions on the LIR under shear stress.

FIGURE 9

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Blood Stored at 4C for 5 Days

- 1.6 % NaCl
- 1.0 % NaCl
- 0.9 % NaCl
- △ 0.8 % NaCl
- ▲ 0.7 % NaCl

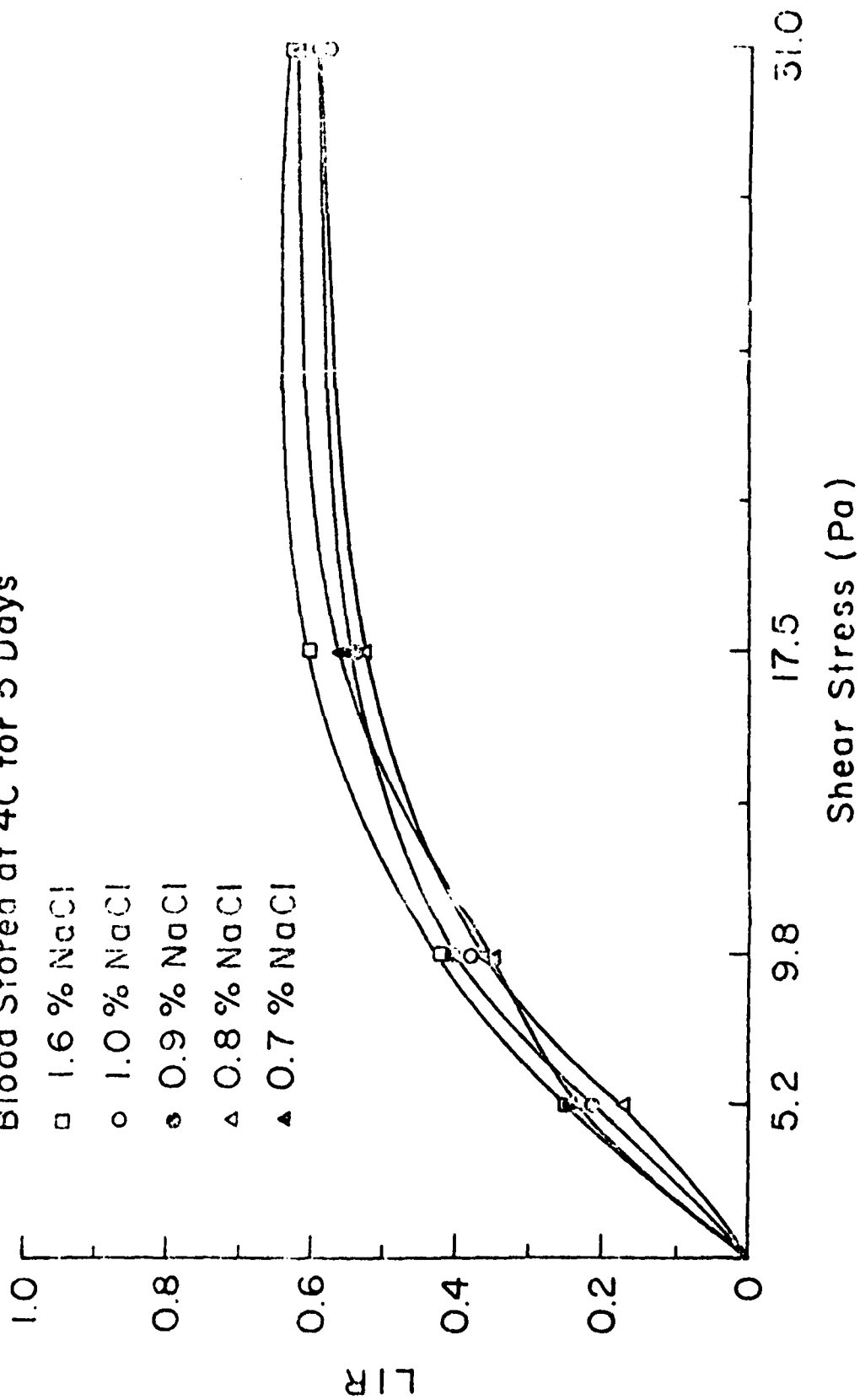


FIGURE 10

The effect of treatment of 20-day-old human red blood cells with various concentrations of NaCl followed by resuspension in isotonic PBS-dextran solutions on the LIR under shear stress.

FIGURE 10

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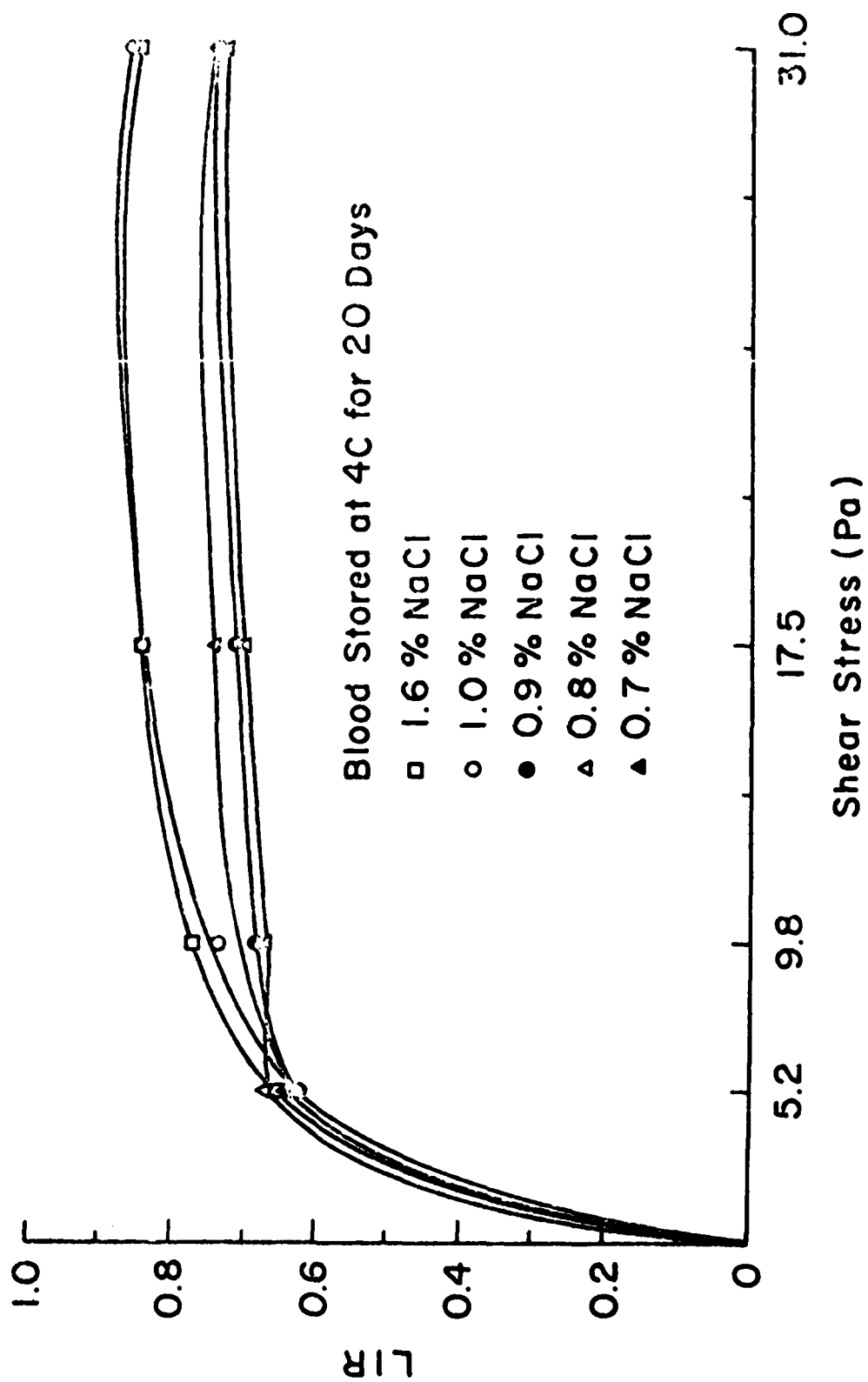


FIGURE 11

Scattergram of the slope of the plot of $1/LIR$ vs. $1/\text{shear stress}$ obtained from human red blood cell samples containing various concentrations of white blood cells. Note the absence of correlation between white blood cell counts and ektacytometry data.

FIGURE 11

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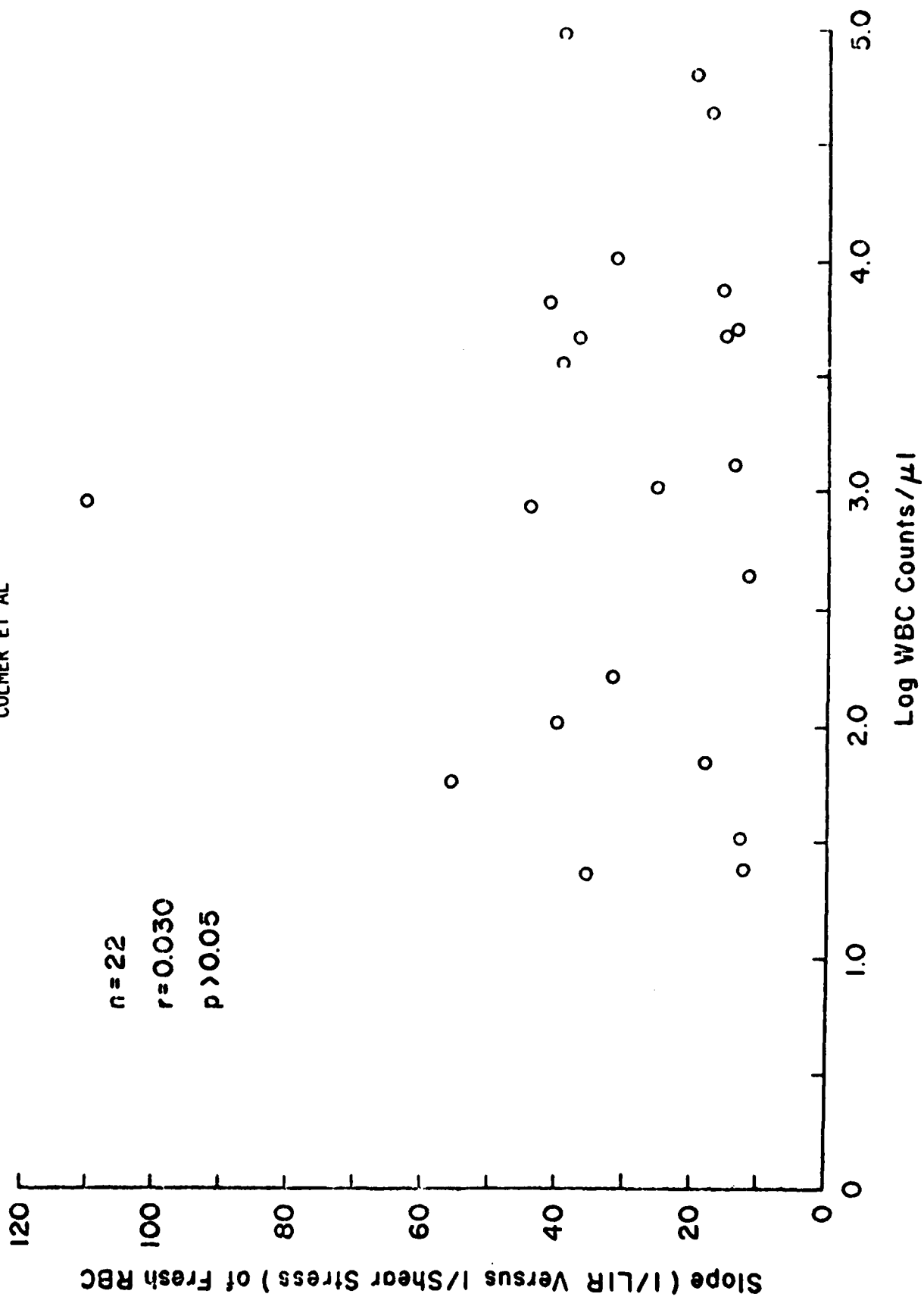


FIGURE 12

Plot of LIR vs. shear stress for fresh and frozen in the presence of 40% glycerol human red blood cells.

FIGURE 12

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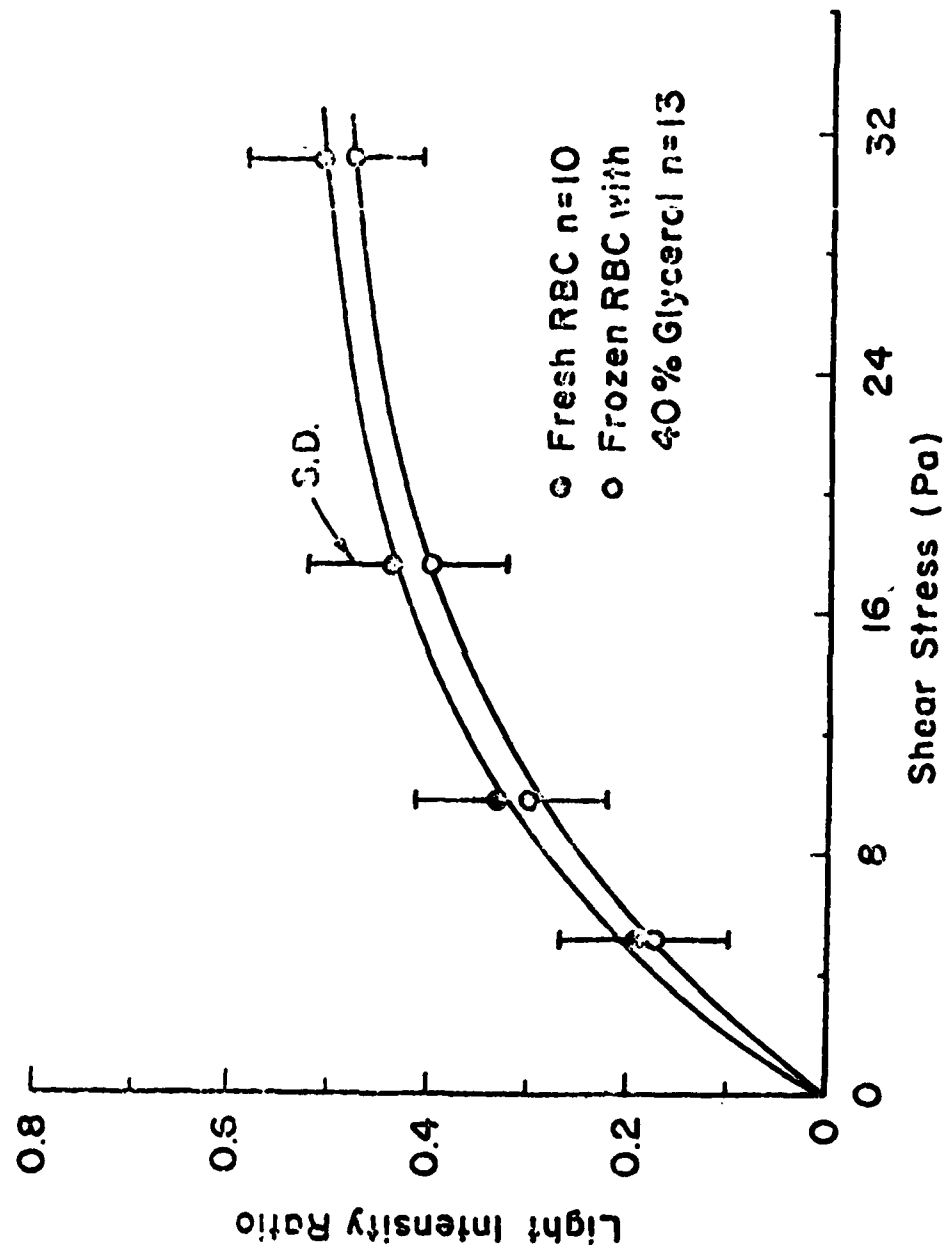


FIGURE 13

Plot of LIR vs. shear stress for phthalate fractionated human red blood cells containing different concentrations of reticulocytes.

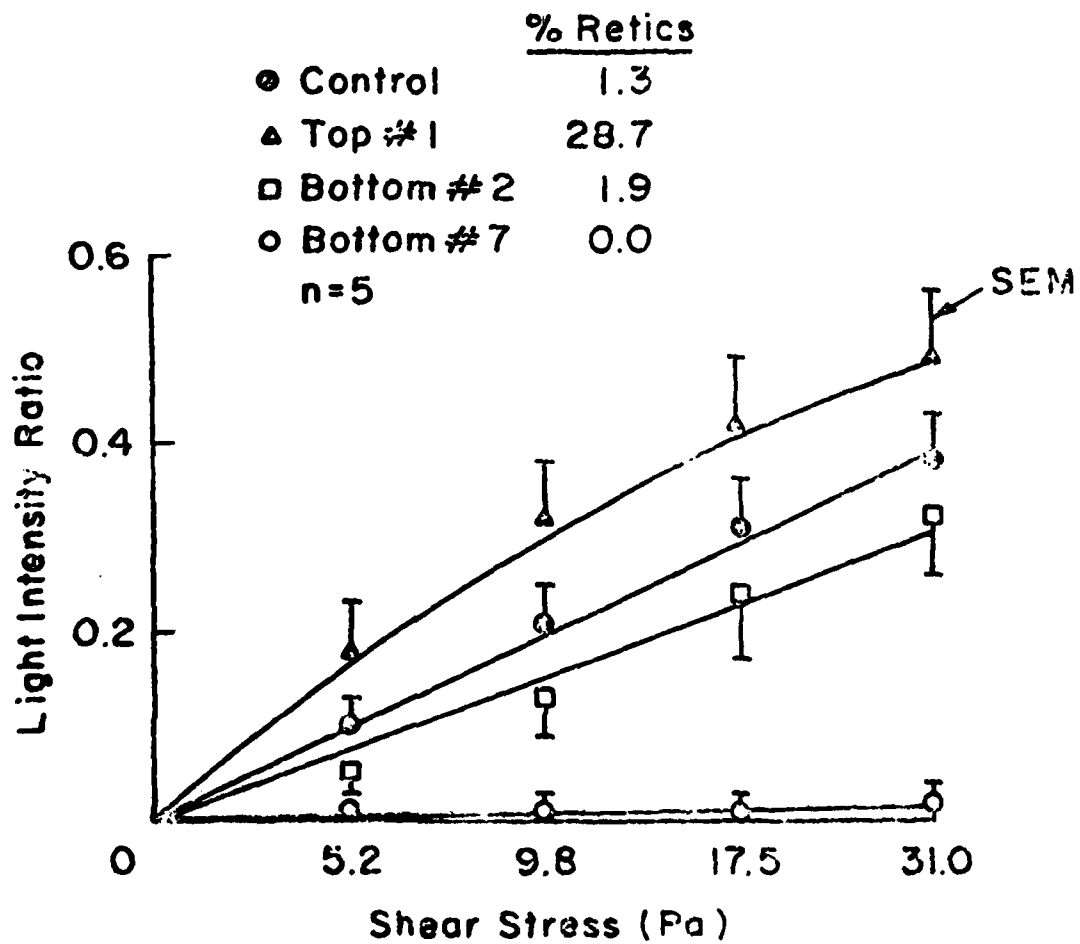


FIGURE 13

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FIGURE 14

Comparison of erythrocyte ektacytometry measurements between neocytes collected on the Fenwal CS-3000 cell separator using the granulocyte chamber and the predonation blood sample.

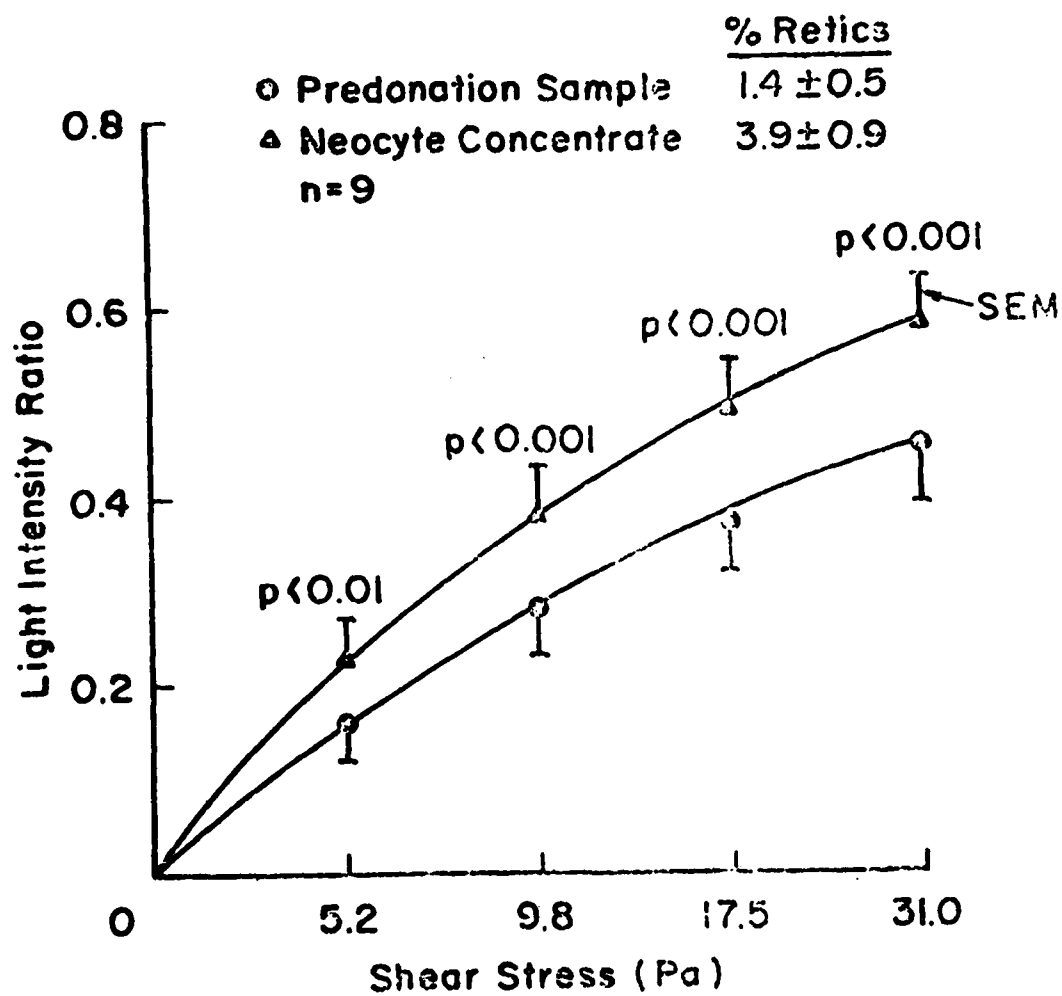


FIGURE 14
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FIGURE 15

Comparison of erythrocyte ektacytometry measurements between neocytes collected on the Fenwal CS-3000 cell separator using the platelet chamber and the predonation blood sample.

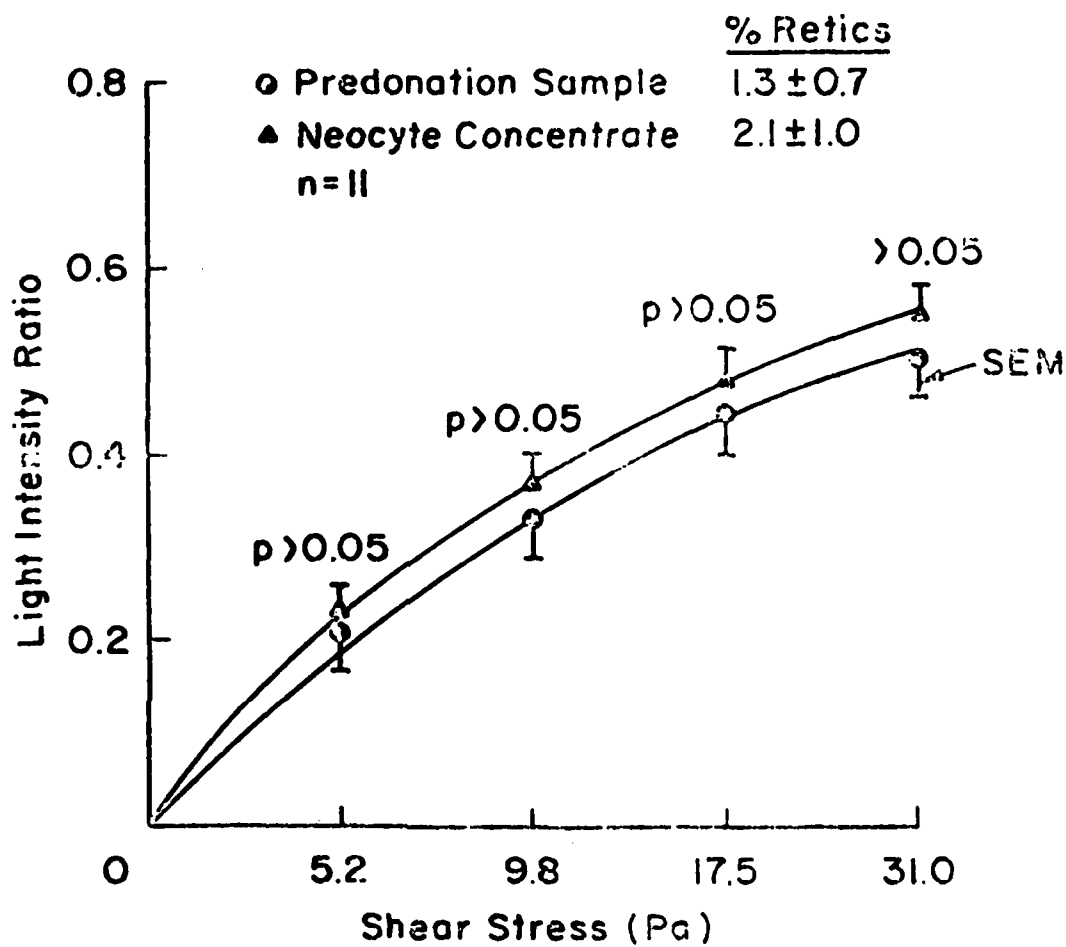


FIGURE 15

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FIGURE 16

Plot of 24-hour posttransfusion survival vs. LIR max for fresh human red blood cells.

FIGURE 16

COLMER ET AL

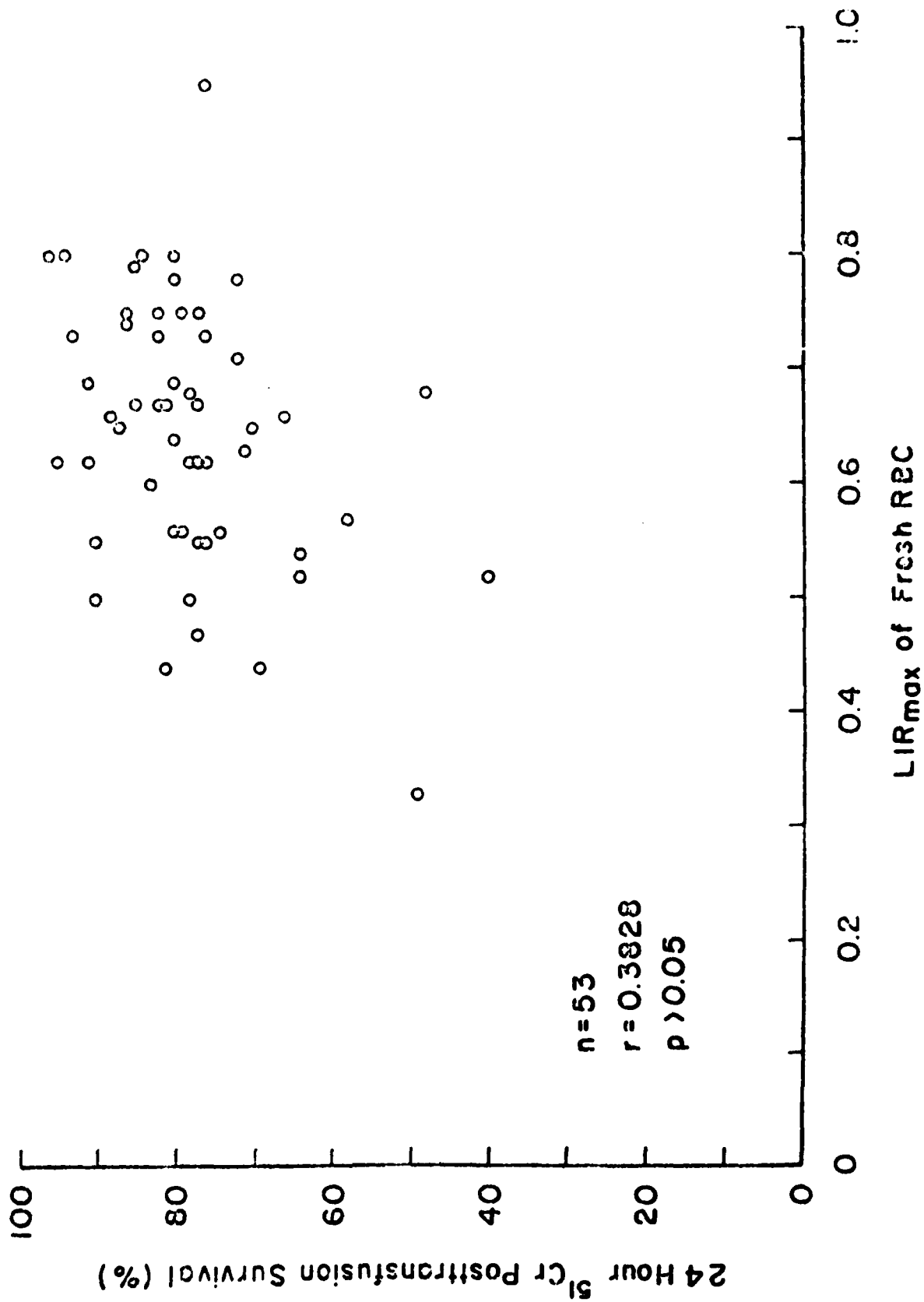


FIGURE 17

Plot of 24-hour posttransfusion survival vs. the log of the slope of $1/LIR$ vs. $1/\text{shear stress}$ of fresh human red blood cells.

FIGURE 17

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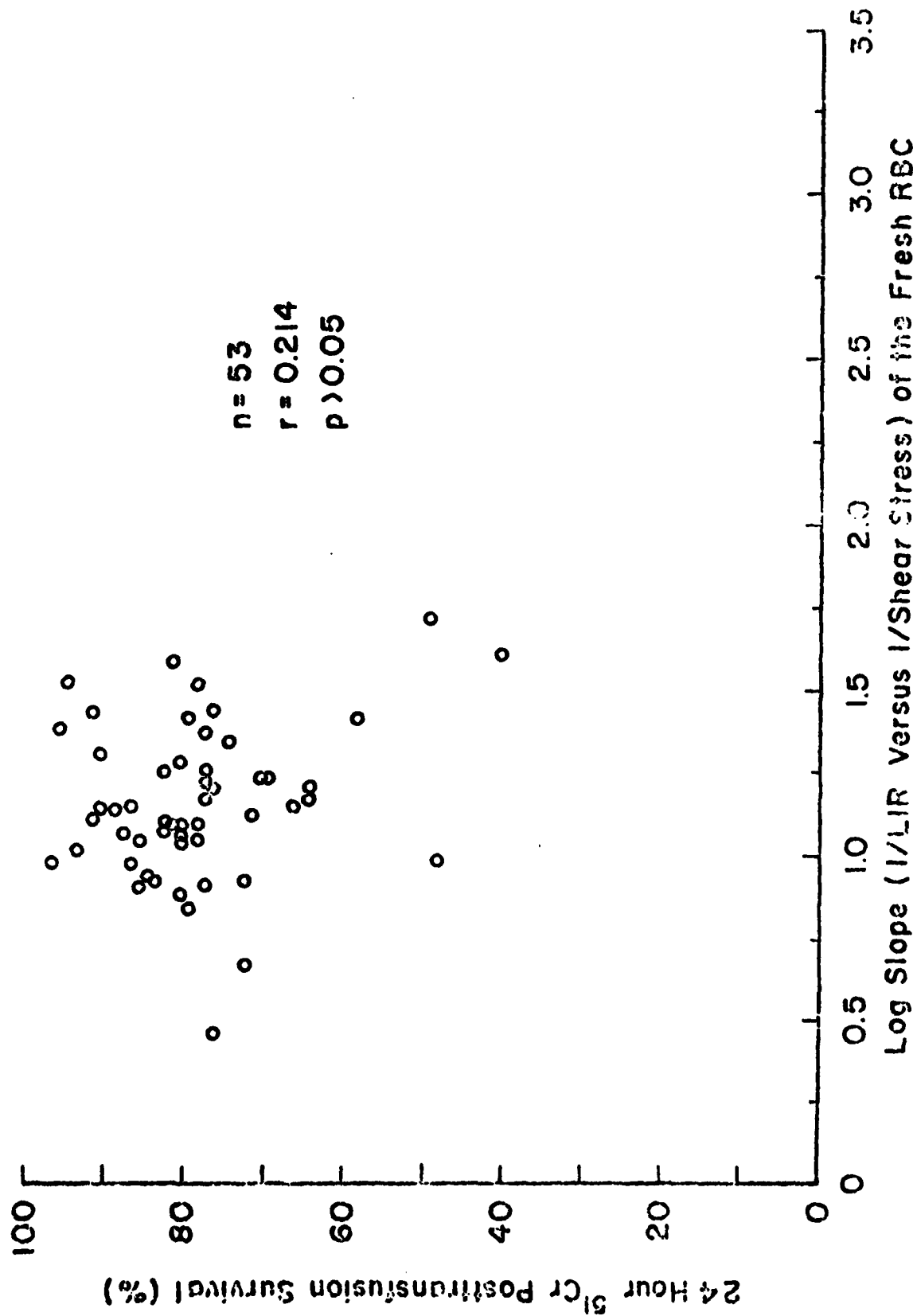


FIGURE 18

Plot of 24-hour posttransfusion survival vs. LIR max for ^{51}Cr -
labeled human red blood cells stored frozen.

FIGURE 18

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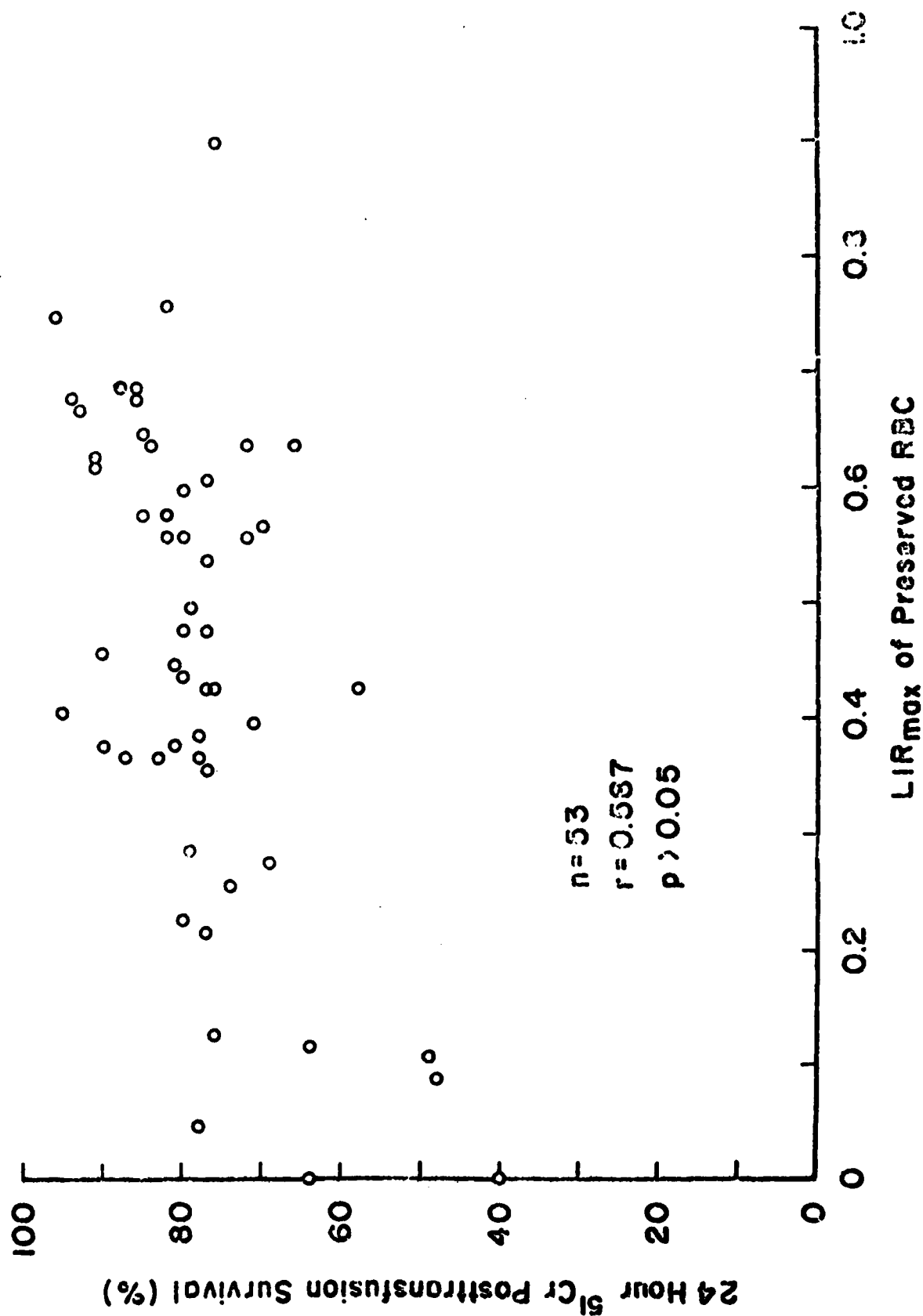


FIGURE 19

Plot of 24-hour posttransfusion survival vs. the slope of $1/\text{LIR}$ vs. $1/\text{shear stress}$ plot for ^{51}Cr -labeled human red blood cells stored frozen.

FIGURE 19

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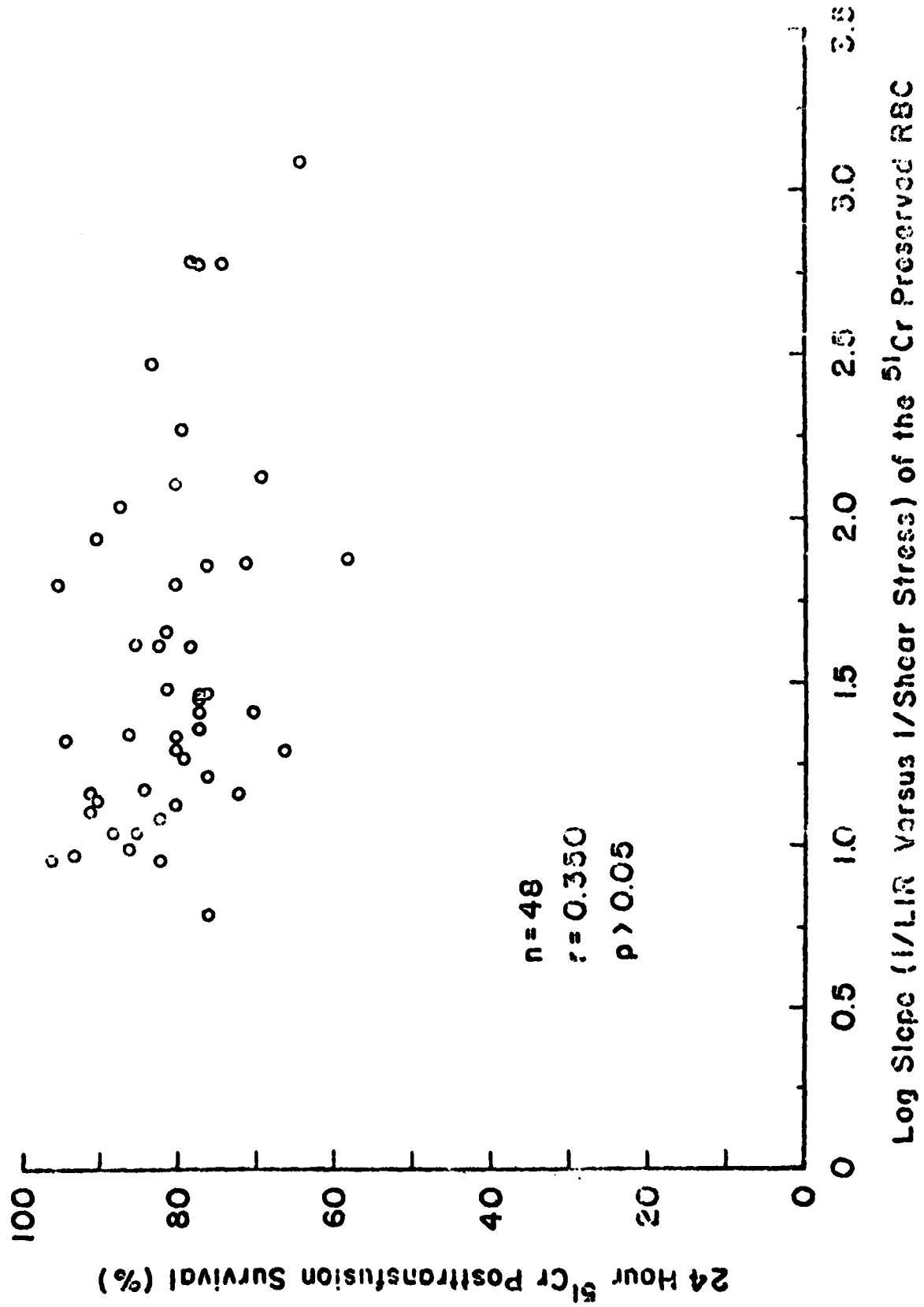


FIGURE 20

Effect of human red blood cell concentrations on the LIR values obtained under variable shear stress conditions (Study I).

Study # 1

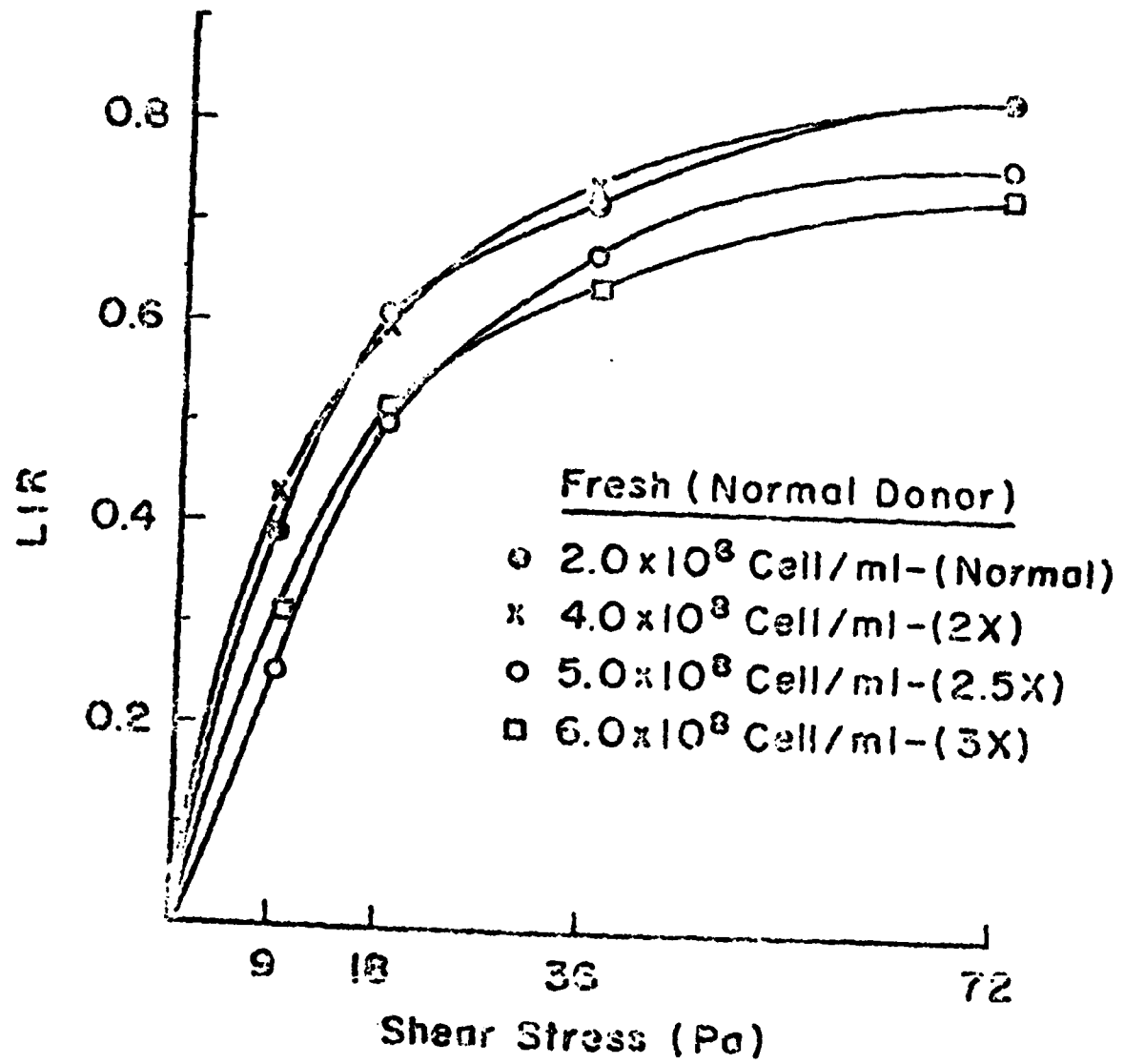


FIGURE 20

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FIGURE 21

Effect of human red blood cell concentrations on the LIR values
obtained under variable shear stress conditions (Study II).

Study #2

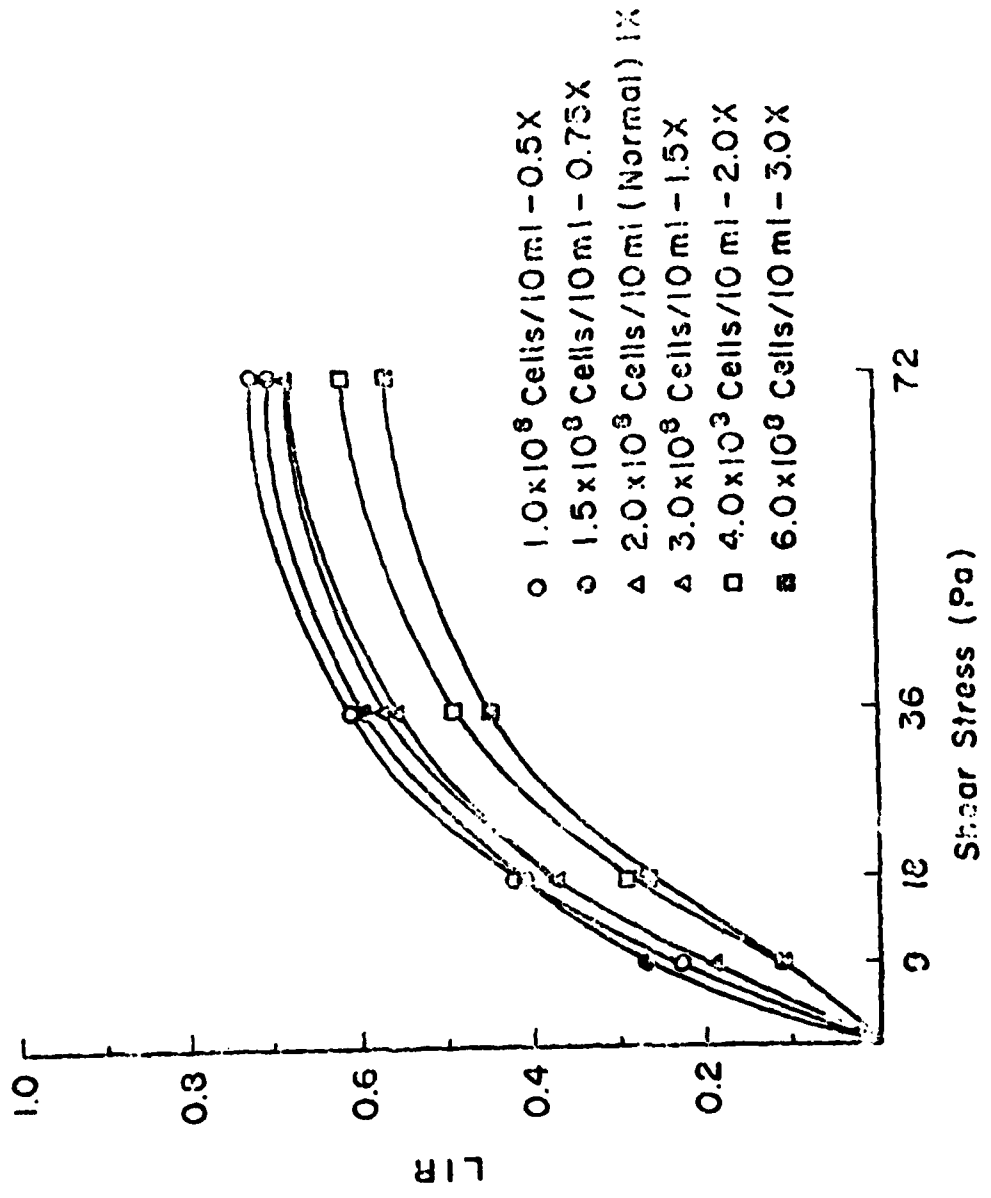


FIGURE 21

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FIGURE 22

Effect of human red blood cell concentrations on the LIR values obtained under variable shear stress conditions (Study III).

Study # 3

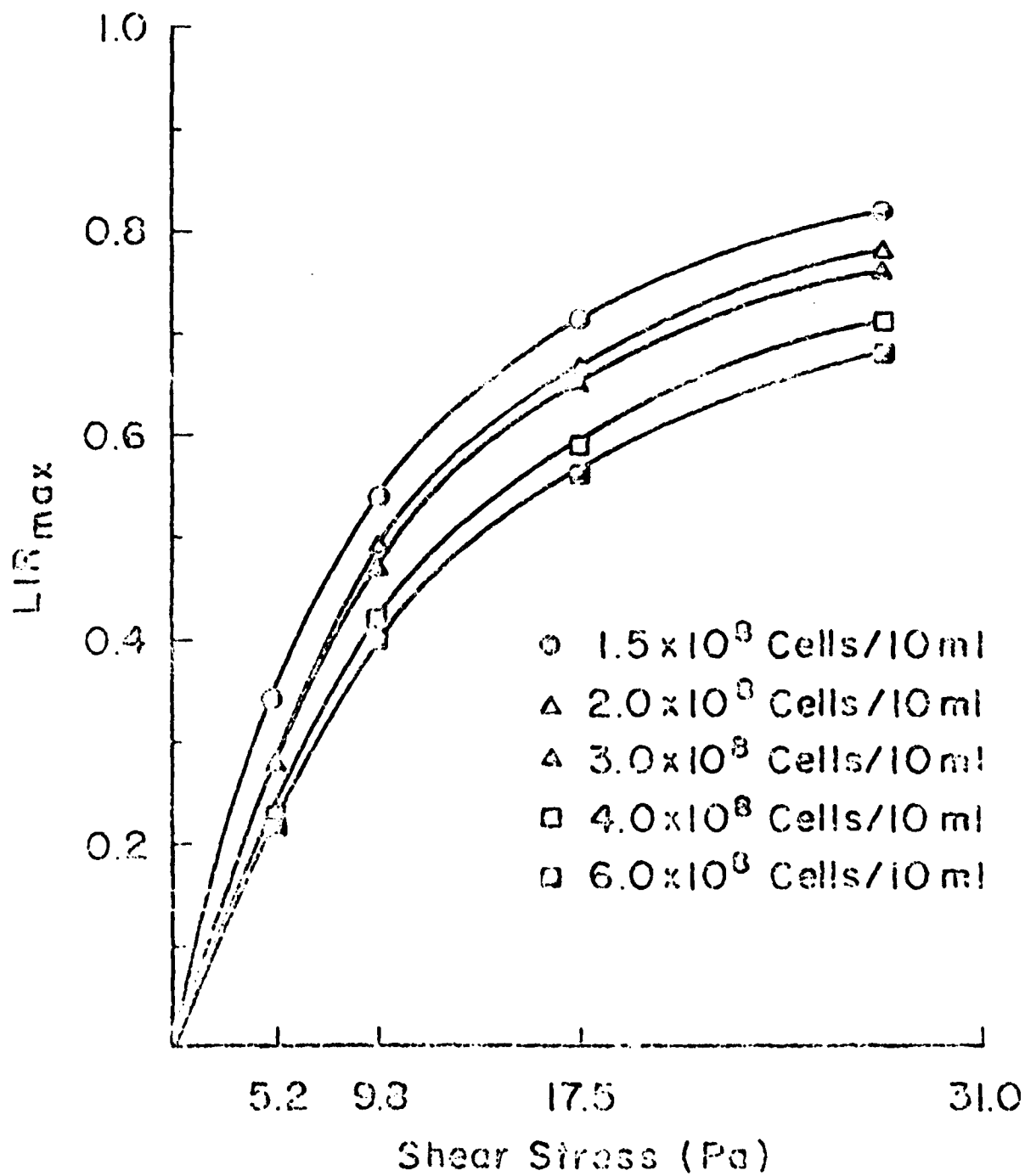


FIGURE 22

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FIGURE 23

A plot showing the linear relationship between LIR max and human red blood cell concentration.

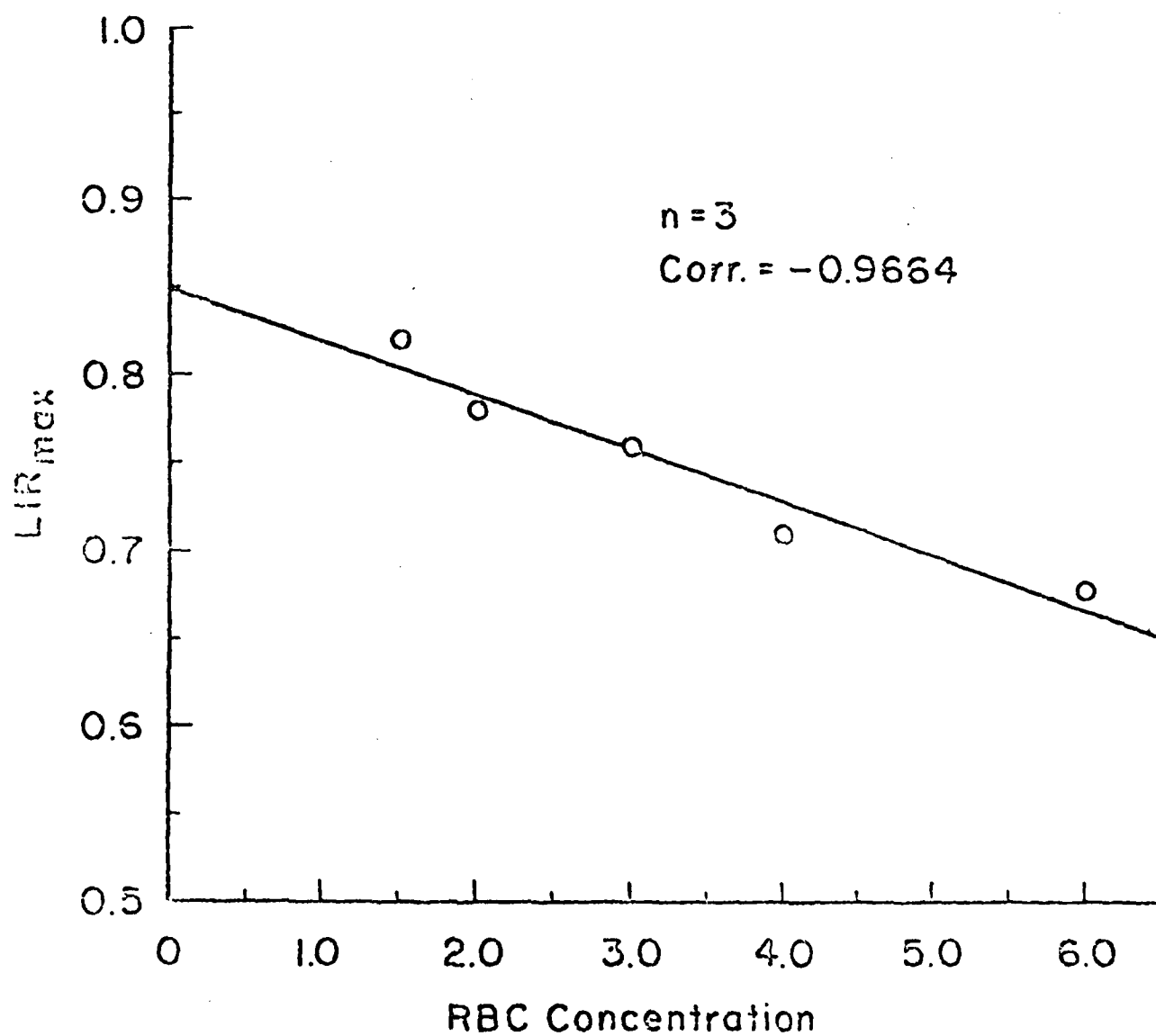


FIGURE 23

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